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Research article

Changes in potato phenylpropanoid metabolism during tuber development

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ABSTRACT

Phenylpropanoid metabolite and transcript expression during different developmental stages were examined in field grown potatoes. Carbohydrate and shikimic acid metabolism was assessed to determine how tuber primary metabolism influences phenylpropanoid metabolism. Phenylpropanoid concentrations were highest in immature tubers, as were some transcript levels and enzyme activities including phenylalanine ammonia lyase (PAL). Phenylpropanoid concentration differences between mature and immature tubers varied by genotype, but in some cases were approximately three-fold. The most abundant phenylpropanoid was chlorogenic acid (5CGA), which decreased during tuber maturation. Hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase (HQT) transcripts were highly expressed relative to other phenylpropanoid genes, but were not well correlated with 5CGA concentrations ($r = -0.16$), whereas HQT enzyme activity was. In contrast to 5CGA, less abundant chlorogenic isomers increased during development. Concentrations of hydroxycinnamic acid amides were higher in immature tubers, as was expression of arginine- and ornithine decarboxylases. Expression of several genes involved in carbohydrate or shikimate metabolism, including sucrose synthase and DAHP, showed similar developmental patterns to phenylpropanoid pools, as did shikimate dehydrogenase enzyme activity. Sucrose, glucose and fructose concentrations were highest in immature tubers. Exogenous treatment of potatoes with sugars stimulated phenylpropanoid biosynthesis, suggesting sugars contribute to the higher phenylpropanoid concentrations in immature tubers. These changes in phenylpropanoid expression suggest the nutritional value of potatoes varies during development.

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1. Introduction

Physiological roles of potato (*Solanum tuberosum* L.) phenylpropanoids include enabling plants to cope with biotic and abiotic stresses [1]. Extensive variance in phenylpropanoid content occurs among potato germplasm, reflecting the considerable genetic diversity of potatoes and the capacity of tubers to synthesize these compounds [2,3]. PAL has been well studied in tubers, and increases in response to light, infection or treatment with elicitor [4,5]. PAL activity and phenylpropanoids increase after wounding, during which a suberized wound periderm is formed [6,7]. Differential expression of phenylpropanoid genes was observed in tubers exhibiting white and purple sectoring [8].

Apart from physiological roles, phenylpropanoids influence the nutritional value of potatoes, with one study showing white potatoes to be the third largest source of dietary phenolics in the American diet [9]. Phenylpropanoids can have antioxidant capacity or other health-promoting characteristics [10–12]. Chlorogenic acid (5CGA, Fig. 1), the most abundant phenolic in potatoes, may

Abbreviations: ADC, arginine decarboxylase; AMY, alpha-amylase; BDCS, bis(-dihydrocaffeoyl)spermine; BDCSD, bis(dihydrocaffeoyl)spermidine; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CP, caffeoyl putrescine; CGA, chlorogenic acid; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; DAP, days after planting; diCGA, dicaffeoylquinic acid; FP, feruloyl putrescine; FQA, feruloylquinic acid; HCT, hydroxycinnamoyl; CoA, shikimate hydroxycinnamoyl transferase; HQT, hydroxycinnamoyl-CoA:quinate hydroxcinnamoyl transferase; INV, soluble acid invertase; Kmp, kaempferol-3-rutinose; ODC, ornithine decarboxylase; PAL, phenylalanine ammonia lyase; PGK, phosphoglycerate kinase; Phe, phenylalanine; PPO, polyphenol oxidase; Rut, rutin; SAG, salicylic acid glucoside; SP, starch phosphorylase; SUSY, sucrose synthase; SSY, soluble starch synthase; TDCS, tris(dihydrocaffeoyl)spermine; TDCSD, tris(dihydrocaffeoyl)spermidine.

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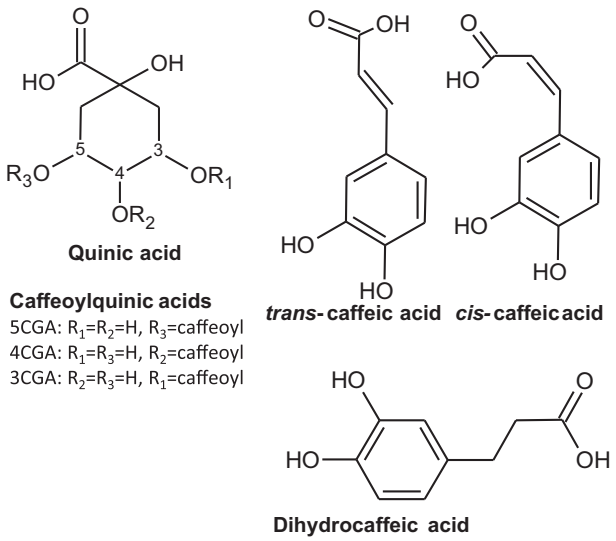

Fig. 1. Structures of hydroxycinnamic metabolites.

have various health-promoting effects, including reduced risk of some cancers, cardiovascular disease [13], hypertension [14], and diabetes [15].

Engineering the tuber phenylpropanoid pathway has the potential to further increase the nutritional value of potatoes [16]. Little is known about phenylpropanoid metabolism in the latter stages of tuber maturation, especially when grown under cropping conditions. A more comprehensive understanding of phenylpropanoid metabolism during tuber maturation in actual field conditions could provide insights about approaches to produce more nutritious potatoes. Because tubers are a staple food consumed in quantity, even minor differences in phenylpropanoid content may be dietarily significant. Hydroxycinnamic acids vary during development in cherries and decreased sharply during fruit development in apples and pears [17,18]. Tuber periderm was reported to have elevated amounts of phenylpropanoids during mid-season bulking and folate was higher in immature tubers [19,20]. Moreover, sucrose stimulates phenylpropanoid expression in plants, and there are large changes in sucrose concentrations during tuber development [21–23]. In this study, we examined whether tuber maturation over the course of the growing season modulates phenylpropanoid expression and explored potential interactions between sugar and phenylpropanoid metabolism as tubers developed from early bulking through to full maturity.

2. Results

2.1. Phenolics during tuber maturation

Phenylpropanoid metabolism was characterized throughout the course of tuber development starting when tubers were ca. 8–28 g (2–3 cm diameter) and ending with fully developed and physiologically mature tubers averaging 180–250 g. The time course of foliar and tuber development, along with tuber specific gravity (dry matter) and carbohydrate (suc, glc, fru) content were analyzed as biochemical indices of tuber maturity (Fig. 2). Collectively, the data in Fig. 2 and Supplemental Table 1 explicitly define the relative physiological maturities of 'Defender', 'Umatilla Russet', and 'Russet Burbank' tubers used for phenylpropanoid analysis at each timepoint throughout the season. Foliar growth was rapid during the first half of the growing season; reaching a maximum at 81–84 DAPS and then declining through 146 DAP for all three cultivars. Increases in tuber yields and average tuber fresh weights were linear through the bulking phase (64–131 DAP) of tuber development (Fig. 2). Tuber growth then slowed appreciably during the maturation phase (ca. 130–170 DAP) of development. Tuber physiological maturity was attained 148, 151, and 146 DAP for 'Defender', 'Umatilla Russet', and 'Russet Burbank' tubers, respectively (Fig. 2).

The concentration of total phenolics was highest at the first developmental stage (60–64 DAP) when 'Defender', 'Umatilla Russet' and 'Russet Burbank' tubers averaged 8–28 g fresh weight (Fig. 3A). Phenolic concentrations fell as tubers developed through to full maturity (stage 10, 170 DAP) in all cultivars. The decrease between stage 1 (64 DAP) and stage 10 (170 DAP) ranged from 16% in 'Defender' to 44% in 'Umatilla Russet.' Phenolic content decreased the most from 64 to 92 DAP, a period consistent with the attainment of maximum foliar development, and then stabilized during the remainder of tuber bulking. A similar trend was evident for 'Green Mountain' tubers. The higher amount of phenolics observed in 'Green Mountain' may be due to both genotypical and environmental components. Concentrations remained high in tubers grown in a different year (data not shown).

The cultivars depicted in Fig. 3A are white-flesh potatoes. To determine whether the overall trend of declining phenylpropanoid concentration with tuber maturity is broadly representative of potato germplasm, five additional genotypes with purple- or yellow-flesh color were examined. Immature potatoes harvested ~75 DAP were compared to mature tubers harvested ~160 DAP from the same location. All five genotypes showed higher concentrations of phenolics in immature tubers (Fig. 3B). The biggest differences in phenolic content between immature and mature tubers were seen in the purple-flesh cultivars, which also had the highest amounts of total phenylpropanoids. The decrease in phenolic compounds during tuber growth occurred on a fresh weight and dry weight basis, indicating that changes were not simply a consequence of starch accumulation during development.

2.2. Shikimate and PAL

To assess changes in phenylpropanoid metabolism during tuber maturation and relationships between secondary and primary metabolism, metabolite pools and expression of key genes within the phenylpropanoid pathway, shikimate or carbohydrate metabolism were measured (Fig. 4). PAL catalyzes the first committed step in the biosynthesis of most phenylpropanoids by converting phenylalanine derived from the shikimate pathway to trans-cinnamic acid and ammonia [24]. Unlike total phenolics, concentrations of phenylalanine were relatively stable during tuber development in three of the four genotypes and did not show a clear trend to decrease (Fig. 5A). PAL activity is regulated transcriptionally and post-transcriptionally [25,26]. In contrast to phenylalanine concentrations, PAL enzyme activity and mRNA levels were higher earlier in tuber development (Fig. 5B and C), which is consistent with the more active phenylpropanoid metabolism observed in immature tubers.

The shikimate pathway can direct carbon flow into phenylpropanoid metabolism by providing the precursors for phenylalanine biosynthesis. Moreover, the shikimate pathway can provide additional precursors for hydroxycinnamic acid biosynthesis (Figs. 1 and 4) when partitioned toward quinate metabolism at a branch point catalyzed by the bifunctional enzyme quinate dehydrogenase, which has shikimate dehydrogenase and dehydroquinate dehydratase activity. Except for the 90 DAP samples, shikimate dehydrogenase enzyme activity, showed a trend to decreasing activity during development (Fig. 5D). Total protein in multiple genotypes was consistently about 2-fold higher in immature tubers. Consequently, when enzyme activity is normalized on a per mg protein basis, this tends to attenuate the

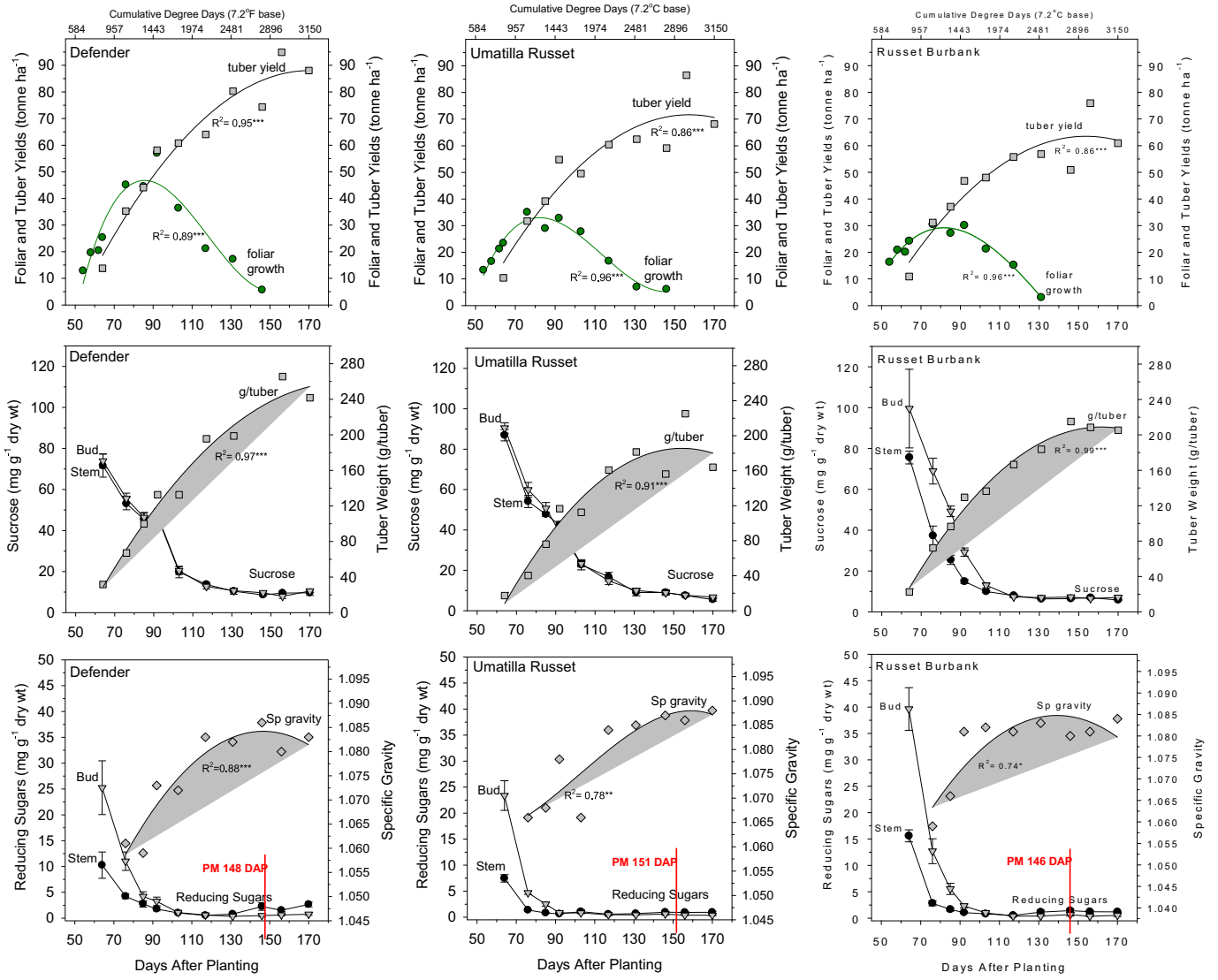

Fig. 2. Foliar and tuber growth (top row) of Defender, Umatilla Russet, and Russet Burbank under late-season management at Othello, WA during 2005. Changes in sucrose concentration, average tuber wt. (middle row), reducing sugars (glucose and fructose), and specific gravity (bottom row) were analyzed to fully define the developmental stages of tubers subsequently used for phenylpropanoid analysis. Tuber physiological maturity (PM) was estimated at 148-, 151-, and 146-DAP for Defender, Umatilla, and Russet Burbank, respectively (bottom row). PM was defined as the average DAP to achieve maximum tuber yield and specific gravity, and minimum sucrose and reducing sugar concentrations in tubers (based on polynomial models). Cumulative degree days at the corresponding days after planting are shown (top row). Foliar, tuber yield and average tuber wt. (g/tuber) data are the average of four replicates of four plants harvested at each sampling. Sucrose, reducing sugars, and specific gravity were determined on four replicates (5 tubers/replicate) at each harvest date (each point is the average of 20 tubers).

developmental effects. When expressed on a per mg tuber dry weight basis, shikimate dehydrogenase and PAL activities were clearly highest in immature tubers relative to mature tubers (data not shown).

2.3. Hydroxycinnamic acids during tuber development

The most abundant soluble phenylpropanoid in these potatoes was the hydroxycinnamic acid, chlorogenic acid (5CGA; 5-O-caffeoylquinic acid), which decreased during tuber development (Fig. 6A), paralleling the decrease seen in total phenolics. The developmental decrease in hydroxycinnamic acids occurred whether expressed on a fresh weight or dry weight basis. Considerably lesser amounts of three additional hydroxycinnamic acid monoesters, 3-O-, 4-O- and cis 5-O-caffeoylquinic acid were present (Fig. 6A) and identified based on MS^2 data and retention times [27,28]. Like 5CGA, cis 5-O-caffeoylquinic acid decreased during development. However, both 3-O- and 4-O-caffeoylquinic acid increased during development in all four genotypes (Fig. 6A). One genotype had small amounts of a dicaffeoylquinic acid that increased during development, as did caffeic acid in the four genotypes. These other hydroxycinnamic acids were considerably less abundant than 5CGA and so would not offset the overall decrease in phenylpropanoids seen during tuber maturation. A negative correlation was observed between the accumulation of 5CGA with the 3- and 4-CGA isomers, and with two other minor hydroxycinnamoyl derivatives (Figs. 6 and 7).

2.4. Phenylpropanoid gene expression and enzyme activity

In *Solanaceous* plants, 5CGA is thought to be synthesized by hydroxycinnamoyl-CoA:quinate hydroxcinnamoyl transferase (HQT), although additional pathways may contribute to its synthesis [29,30]. Despite the decrease observed in 5CGA, qRT-PCR

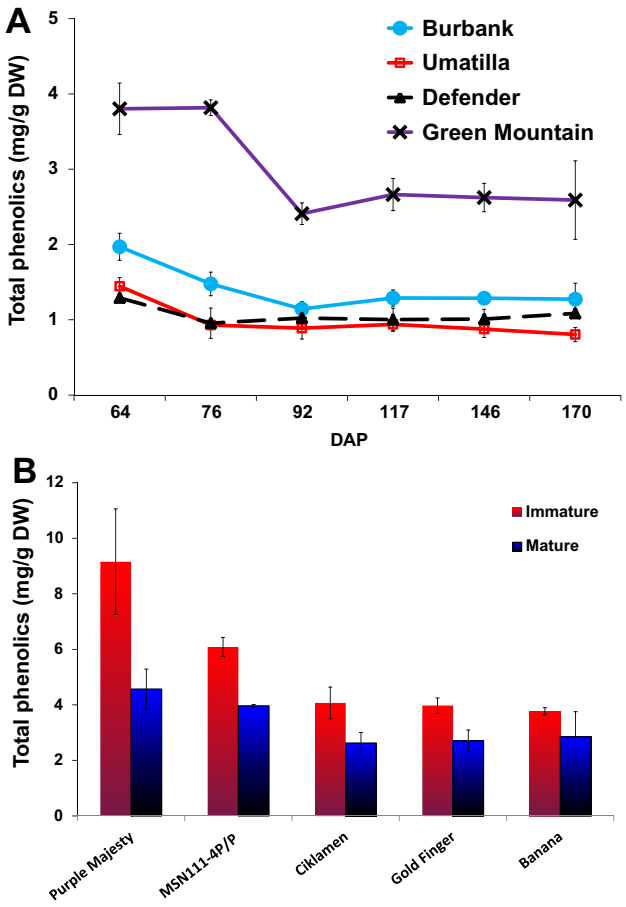

Fig. 3. Total phenolics in potatoes. (A) Tubers from four genotypes harvested at six developmental stages (see Fig. 2) throughout the growing season. (B) Tuber total phenolics in immature (~75 DAP) or mature tubers (~160 DAP) from five additional genotypes. For each timepoint, the average of 3 biological replicates is shown with standard deviation. Days after planting (DAP) is shown for Umatilla Russet, Defender and Russet Burbank; DAP for 'Green Mountain' is instead from 60 to 110 days in 10 day increments.

analysis did not reveal a clear decrease in HQT transcript levels, which were instead relatively stable throughout development and did not correlate with 5CGA (Figs. 6B and 7). Although HCT (hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase) is further upstream in 5CGA biosynthesis and was expressed at lower levels in tubers than HQT, a better correlation was seen between 5CGA levels and HCT expression ($r = 0.82$; Figs. 6 and 7) than with HQT. Both HCT and PAL expression correlated with overall phenylpropanoid concentrations during development, which showed a dip into midseason, followed by a slight increase as tubers reached physiological maturity.

Three additional phenylpropanoid genes, C4H, C3H and CHI, were more strongly expressed in immature than mature tubers (Fig. 8). C4H and C3H showed similar expression to PAL, decreasing in mid-season as the tubers bulked before increasing into maturity. CHI expression was the most variable. The relative abundance of PAL, HCT, CHI, and C4H transcripts was roughly comparable, while HQT was the most highly expressed and C3H the least expressed. Expression of most of these genes showed correlations with total phenolics (Figs. 7 and 8) C3H, $r = 0.86$; C4H, $r = 0.65$; CHI, $r = 0.32$; PAL, $r = 0.91$; HQT, $r = -0.32$; HCT, $r = 0.86$.

Because 5CGA is the most abundant soluble phenylpropanoid in white potatoes and alternative pathways are possible, we sought to use transient assays to confirm whether HQT was a major source of 5CGA. Potato HQT was cloned and used to make a silencing construct, which was then introduced into potato leaves via Agro-infiltration to knockdown HQT expression. However, it was not possible to achieve good infiltration of potato leaves, so tobacco was used instead. Potato HQT was 87% homologous with that from tobacco. 5CGA concentrations 7 days post-infiltration in empty vector inoculated leaves were 0.91 μmol/g (±0.06 μmol/g) versus 0.58 μmol g^{-1} (±0.13 μmol g^{-1}) in the silenced leaves, a ~37% decrease.

HQT enzyme activity was not detected in crude tuber extracts, so a 30–80% ammonium sulfate fraction was used to examine the HQT forward reaction. Caffeoyl-CoA was metabolized in an enzyme dependent manner in tuber extracts; however, this activity was not dependent on quinic acid, suggesting other enzyme(s) besides HQT were responsible. Therefore, the reverse reaction was used and the 5CGA-dependent formation of caffeoyl-CoA from chlorogenic acid measured. Immature tubers had over 2-fold more HQT enzymatic activity (Fig. 6C) than mature tubers. Chlorogenic acid can be oxidatively degraded by polyphenol oxidase (PPO), which was also higher in immature tubers (Fig. 6C).

2.5. Additional tuber phenylpropanoids

Additional compounds were measured in tubers including ferulic acid derivatives, hydroxycinnamic acid amides (HCAAs) and salicylic acid (Supplementary Figs. 1 and 2). Not all compounds were detected in all genotypes and most of the compounds decreased with tuber maturation. Caffeoyl putrescine was the most abundant polyamine derivative detected and decreased during tuber maturation. Similar trends were seen for four dihydrocaffeoyl polyamines, of which bis(dihydrocaffeoyl) spermine and tris (dihydrocaffeoyl) spermine were the most abundant. Two feruloyl putrescine isomers were present in 'Green Mountain,' and these decreased during maturation.

Few of the potato genes involved in HCAA metabolism have been identified, so expression of genes that mediate entry into the putrescine pathway was measured (Fig. 4). In plants, putrescine can be synthesized from arginine decarboxylase (ADC) or ornithine decarboxylase (ODC) [31]. Arginine is the major polyamine precursor in *Nicotiana* [32]. Two ADC isomers were found in database searches. ADC1 in particular was expressed at the highest levels in mature tubers (Fig. 8). ODC expression decreased during maturation and expression was considerably lower than ADC. Similar results were seen when using different ODC and ADC primers (data not shown). While most of the HCAAs did show a rise into the later developmental stages compared to mid-maturation, none showed greater amounts in mature tubers than immature tubers and they only weakly correlated with ADC expression.

Soluble phenylpropanoids might be expected to be more nutritionally relevant than insoluble ones and the compounds measured above are soluble phenylpropanoids extracted in aqueous methanol. Developmental changes in insoluble phenylpropanoids are also of interest and a large percentage is used for lignin biosynthesis. Lignin was measured in Green Mountain at each developmental stage and ranged from 1.13 to 1.48 mg/g DW, but no obvious trend was observed ($p = 0.28$).

2.6. Carbohydrate and shikimate gene expression

Foliar growth peaked around 80–90 DAP, reflecting the change in source/sink relationships to favor tuber growth over foliar development (Fig. 2). Tuber physiological maturity (PM) did not occur until 146–151 DAP, well after foliar growth peaked. Sucrose, fructose and glucose concentrations were markedly higher in immature tubers and decreased with maturity (Fig. 2). Thus,

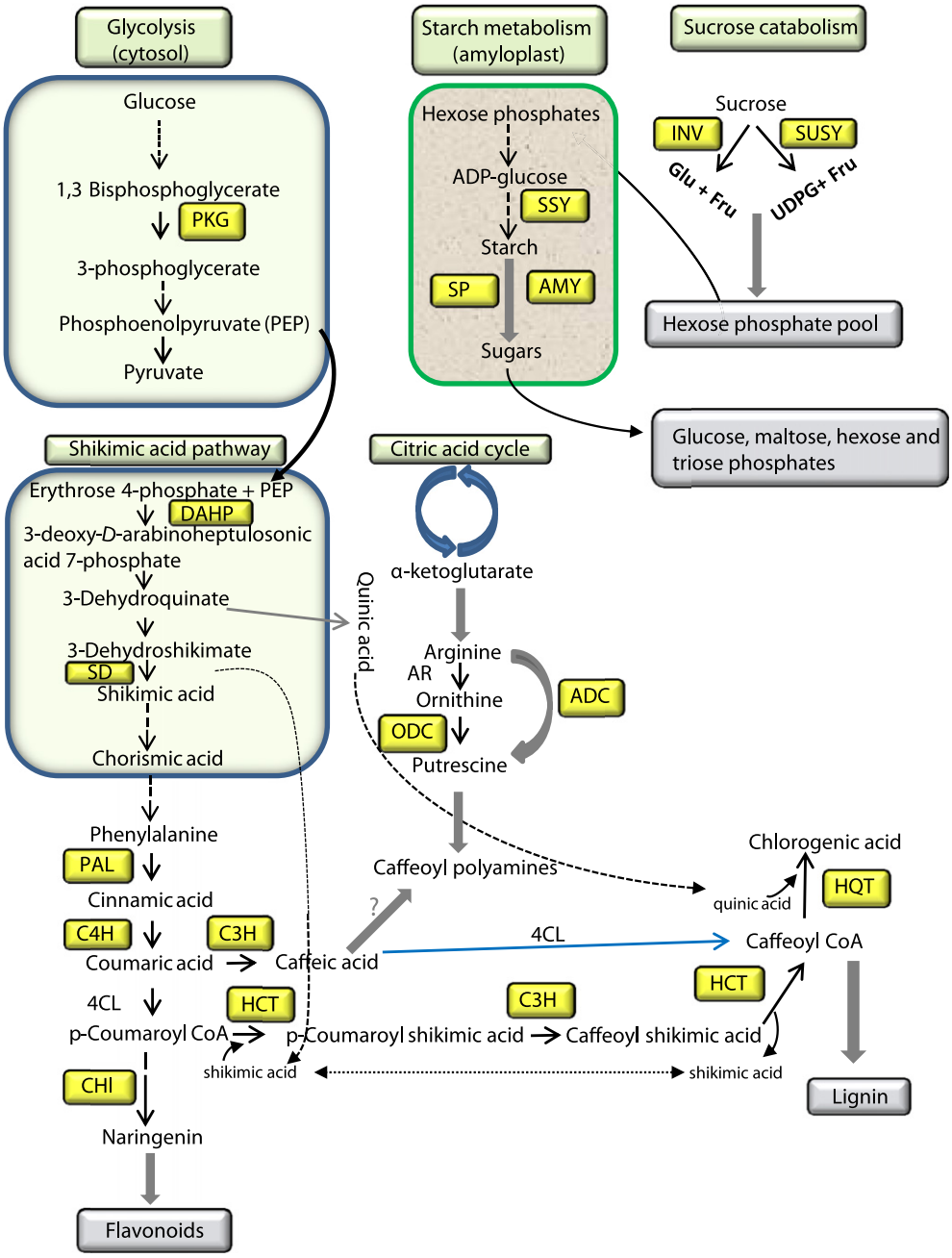

Fig. 4. Carbohydrate and phenylpropanoid pathways. Expression of genes highlighted in yellow was measured. Colored arrows represent a minor pathway. Gray arrows represent multiple enzymatic steps not shown. Dashes indicated the number of enzymatic steps between compounds. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

phenylpropanoid concentrations were highest when sugars were highest. To examine relationships between phenylpropanoids and primary metabolism during tuber development, the expression of nine genes (Supplemental Table 3) in carbohydrate or shikimate metabolism were analyzed. Phenylalanine and its phenylpropanoid derivatives, along with tyrosine and tryptophan are derived from the shikimic acid pathway, so the developmental expression of the gene encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase, which catalyzes the committed step of the shikimate pathway, was measured. Searches identified two isoforms, of which DAHP1 was the most highly expressed (Fig. 9). DAHP1 was highest in immature tubers, decreased during development before rising again into maturity. This trend was similar to that seen in a majority of the phenylpropanoid genes (Fig. 8). Unlike DAHP1, no developmental trend was seen for DAHP2. Moreover, as opposed to DAHP expression, phenylalanine concentrations were relatively stable (Fig. 5A), whereas tryptophan and tyrosine showed a trend to increase (data not shown).

Flux through the shikimate pathway depends on carbohydrate metabolism. Sucrose concentrations decreased throughout development (Fig. 2), as did expression of sucrose synthase (SUSY), an enzyme that also has sucrolytic activity (Fig. 9A). Five starch metabolism genes were measured, of which SUSY was the second most highly expressed gene, markedly more so than invertase

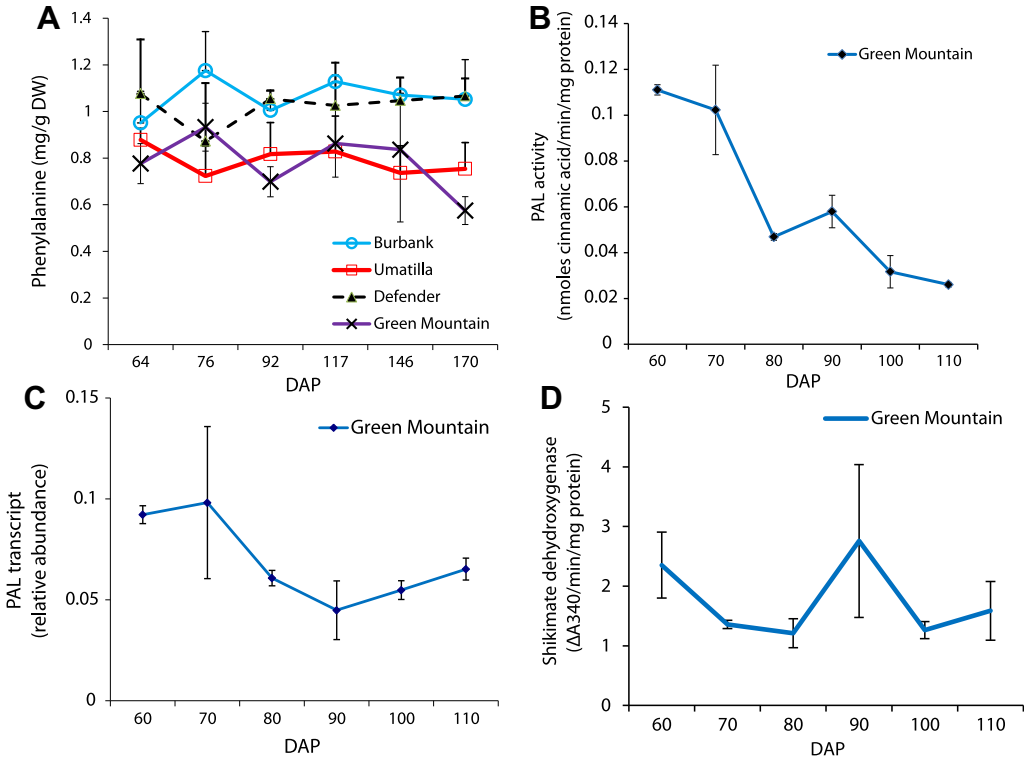

Fig. 5. Tuber phenylalanine metabolism. (A) Phenylalanine concentrations in four genotypes at different developmental stages. Days after planting (DAP) is shown for Umatilla Russet, Defender and Russet Burbank; DAP for 'Green Mountain' is instead from 60 to 110 days in 10 day increments. (B) PAL enzyme activity or gene expression (C) in 'Green Mountain' tubers. For each timepoint in (A) and (B), the average of 3 biological replicates is shown with standard deviation. QRT-PCR data represents the averages of 3 biological replicates with two technical replicates each. (D) Shikimate dehydrogenase activity in 'Green Mountain.'

(INV), a second sucrolytic enzyme. Like SUSY, INV also was highest in immature potatoes and decreased during development. A similar trend was observed for soluble starch synthase. The most highly expressed gene was starch phosphorylase, which was relatively stable during development. The only gene that exhibited a marked developmental increase was α-amylase, which liberates sugars from amylose and amylopectin. In addition to the starch metabolism genes, expression of two isoforms of phosphoglycerate kinase, a glycolysis pathway gene, were measured and both showed a gradual decrease during tuber maturation, especially PGK2 which was 2–3-fold more highly expressed than PGK1.

These results suggested a correlation between sugar and phenylpropanoid metabolism during tuber maturation. To more directly assess the effects of sugars on phenylpropanoid biosynthesis, tuber discs were incubated in sucrose or glucose solutions for 24 h. 5CGA concentrations increased over four fold in glucose treated samples and phenylalanine decreased (Fig. 9B). Complicating interpretation was that wounding was a strong inducer of phenylpropanoids as seen in the differences between untreated slices at time zero versus 24 h after wounding. Wounding alone decreased the phenylalanine pool and increased some downstream phenylpropanoids (Fig. 9B). Relative to wounding alone, glucose feeding showed a trend to further increase 5CGA and CGA isomers, along with kaempferol-3-O-rutinoside which was not detectable in the time zero control. To avoid the stimulatory effects of wounding, potato tissue culture plantlets were grown in standard media or media supplemented with 150 mM sucrose (Fig. 9C). Treatment with 150 mM sucrose induced a substantial increase of the major chlorogenic acid isomers and a smaller increase in caffeoyl putrescine. The flavonoid kaempferol-3-*O*-rutinoside was strongly induced by sucrose. The amounts of the shikimate derived amino acids tryptophan and tyrosine decreased.

3. Discussion

Previous studies on metabolic changes during tuber development typically were greenhouse studies concentrating on carbohydrate metabolism during the earliest stages of tuber development, from non-tuberized stolons through tuberization to immature tubers [33–35]. The study reported herein differs by focusing on phenylpropanoid metabolism during the normal course of tuber growth from immature to fully developed and physiologically mature tubers under field conditions. A key finding is that there were substantial changes in phenylpropanoid metabolism accompanying tuber ontogeny through the growing season. Importantly, conducting this analysis in the field instead of the greenhouse ensures results are relevant to the actual crop. Field plants are typically more vigorous and healthy than greenhouse grown potatoes. It is especially difficult (if not impossible) to produce mature, healthy plants and fully developed tubers in the greenhouse that remotely approximate those from the field in yield, size, basal metabolism, and physiological maturity. A study silencing asparagine synthase in potatoes demonstrated how different the results can be between greenhouse and field, highlighting how little relevance greenhouse trials sometimes have for the crop [36]. In our experience, marked differences exist between phenylpropanoid profiles of potatoes grown in the greenhouse or the field; for example, Fig. 10 shows a purple genotype grown in the field versus greenhouse. Hence, the study reported herein is unique in detailing for the first time how phenylpropanoid metabolism is modulated in tubers during ontogeny under field conditions.

Maturation had a strong effect on tuber phenylpropanoid pools, which were higher in immature tubers, and associations were observed between phenylpropanoid, carbohydrate and shikimate metabolism. Differences cannot be solely attributed to developmental

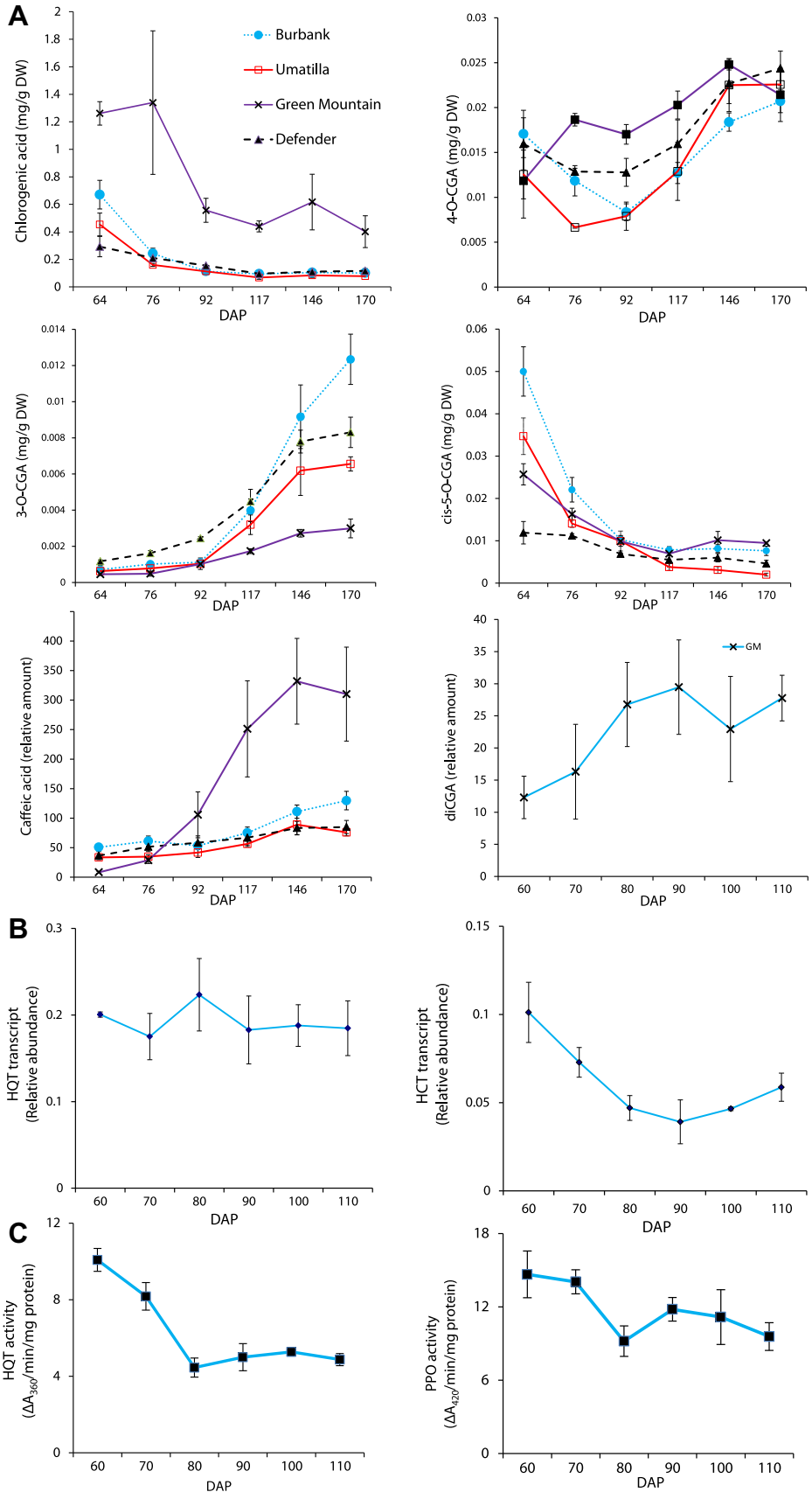

Fig. 6. Tuber hydroxycinnamic acid expression. (A) Amounts of six hydroxycinnamic acids in tubers at different stages. Days after planting (DAP) is shown for Umatilla Russet, Defender and Russet Burbank; DAP for 'Green Mountain' is instead from 60 to 110 days in 10 day increments. For each timepoint, the average of 3 biological replicates is shown with standard deviation. The legend is shown in the top left panel (B) relative amounts of HQT (left) or HCT (right) transcripts in 'Green Mountain' tubers at six different developmental stages. QRT-PCR data represents the normalized averages of 3 biological replicates with two technical replicates each. (C) HQT (left) and PPO (right) activity in 'Green Mountain'. Average of 3 biological replications with standard error are shown.

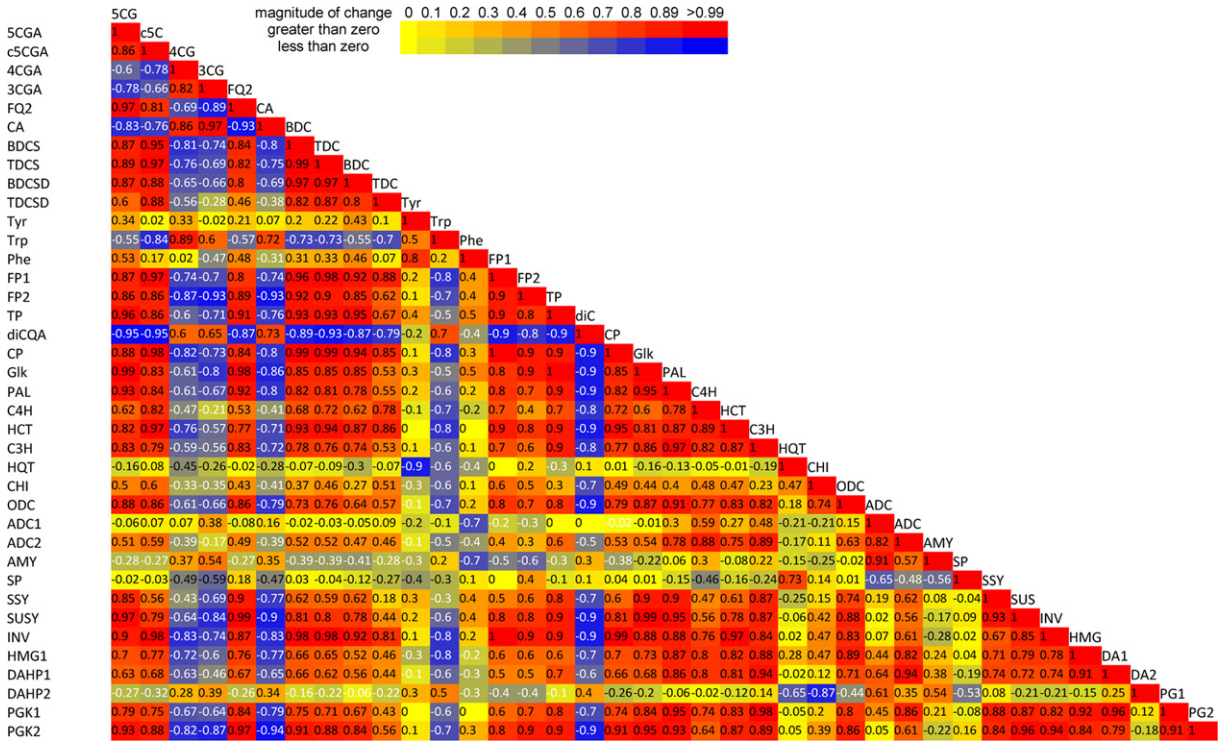

Fig. 7. Correlation analysis of phenylpropanoid and carbohydrate transcript and metabolite levels in tubers. Pearson correlation coefficients were calculated and used to generate a heatmap in which positive correlations are shown in red and negative correlations in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

changes; some could be due to environmental effects. However, multiple genotypes were collected over four different years, from multiple fields in different states and all showed the trend of higher concentrations in immature tubers. Additionally, one of the cultivars was grown in four different fields over two years and showed a similar developmental decrease in each (data not shown). While it is likely that the absolute amount of each phenylpropanoid will vary between years or locations due to environmental factors, results suggest the higher concentrations found in immature tubers have a developmental component. Glycoalkaloid content was also higher in immature tubers and showed a strong positive correlation with phenylpropanoid content (Fig. 7 and Supplementary Fig. 1). However, it is not clear whether there is a causal relationship between phenylpropanoids and glycoalkaloids, which are derived from the mevalonate pathway.

A major contributor to the elevated phenylpropanoid content in immature tubers was 5CGA. Curiously, transcript abundance of almost all the other phenylpropanoid and carbohydrate genes better correlated with 5CGA pools than did HQT (Fig. 7). HQT knockdown transient assays supported its involvement, and HQT enzyme activity was higher in immature tubers, over 4-fold higher if expressed on a tuber weight basis. This suggested HQT expression was not regulated at the transcriptional level, but HQT enzyme activity was a key determinant of developmental changes in tuber phenylpropanoid content. The changes in the neutral HCAAs during development were interesting, but apart from their role in strengthening cell walls and defense responses, definitive proof of biological function is lacking. Suggested roles include modulating cell division, tuber sprouting [31], tuber acid phosphatase activity [37], pollen development and fertility [38,39], and serving as polyamine storage pools or seed nitrogen reserves [40–42].

One mechanism for the increased phenylpropanoids in immature tubers could be that sucrose, fructose and glucose concentrations were highest earlier in tuber maturation, as was expression of key genes and enzymes in the glycolytic and shikimate pathways. The strong correlation between sucrose and total phenolics during development ($r = 0.91$ in Russet Burbank) and the higher expression of SUSY (Fig. 9) was consistent with a role for sugars in modulating phenylpropanoid synthesis. Sucrose induces phenylpropanoids in grapes, broccoli and *Arabidopsis*, and can affect phenylpropanoid gene expression via MYB transcription factors [23,43,44]. Additionally, sugars provide carbon for phenylpropanoid synthesis, with up to 20% of total plant carbon entering the pathway [45]. Interestingly, the increased expression of some phenylpropanoids later in development paralleled the increase in α-amylase expression that should increase tuber sugar content. Supporting this is Fig. 2, which while difficult to resolve because of the *y*-axis scale, shows reducing sugar (glc + fru) concentrations increased substantially in tubers from late bulking through physiological maturity to harvest. From a low at 117 DAP, reducing sugars increased approximately 3-fold, 1.25-fold, and 2-fold in 'Defender', 'Umatilla Russet', and Russet Burbank' tubers, respectively, through 170 DAP. This shows a relationship with phenolic concentrations, because the late increase in sugars was least in 'Umatilla' as was the change in phenylpropanoids (Fig. 3A). The extent to which these reducing sugar increases modulate changes in phenylpropanoid metabolism during maturation warrants further investigation.

Because of high consumption, staple crops have greater potential to impact health than crops consumed more sparingly. Over a three-fold difference was seen for some phenylpropanoids and up to an approximate two-fold difference was seen in total phenolics between immature and immature tubers from some genotypes. Potatoes with higher amounts of phenylpropanoids reduced inflammation in human feeding studies [46], and immature high-phenylpropanoid potatoes were found to lower blood pressure and raise serum antioxidants [47]. The higher phenylpropanoid

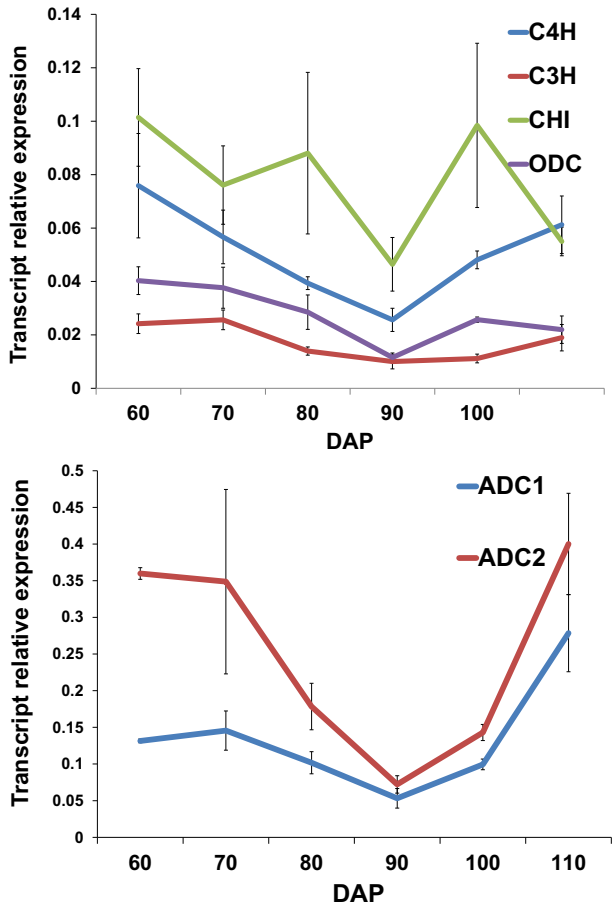

Fig. 8. Relative expression of six genes involved in phenylpropanoid metabolism. 'Green Mountain' tubers were harvested at six developmental stages (DAP). Normalized qRT-PCR data represents the average of 3 biological replicates with two technical replicates each for each timepoint. C4H, cinnamate 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CHI, chalcone isomerase; ODC, ornithine decarboxylase; ADC, arginine decarboxylase.

concentrations in immature potatoes may be relevant for human nutrition because such potatoes are marketed and consumed.

4. Materials and methods

4.1. Chemicals

EDTA and HPLC grade methanol were purchased from Fisher (NJ). HPLC grade acetonitrile, acetic acid, formic acid, and trifluoroacetic acid (TFA) were purchased from J.T. Baker (NJ). Ammonium formate was from Fluka. Chlorogenic acid, gallic acid, caffeic acid, salicylic acid, phenylalanine, sugars, chaconine and solanine were purchased from Sigma (St. Louis, MO).

4.2. Plant material and tuber developmental stages

Potato cultivars were grown at Moses Lake, WA or the Washington State University (WSU) Irrigated Agriculture Research and Extension Center at Othello, WA, except for 'Green Mountain', which was grown at Palmer and Fairbanks, Alaska in 2007 and 2008. 'Russet Burbank,' 'Defender' and Umatilla Russet' were grown in 2005 and samples corresponding to developmental stages 1, 2, 4, 6, 8 and 10 (Supplemental Table 1) were collected on June 16 (64 days after planting, DAP), June 28 (76 DAP), July 14 (92 DAP), Aug 8 (117 DAP), Sept 6 (146 DAP) and Sept 30 (170 DAP) for phenylpropanoid analysis. 'Green Mountain' samples were harvested on July 24 (60 DAP), Aug 3 (70 DAP), Aug 13 (80 DAP), Aug 23 (90 DAP), Sept 2 (100 DAP), and Sept 12 (110 DAP). 'Purple Majesty', MSN111-4PP (each purple flesh) and 'Ciklamen,' 'Gold Finger' and 'Banana' (all yellow) were grown in under standard commercial conditions for the Columbia Basin in 2010 with immature tubers harvested ~60–75 DAP and mature tubers ~160 DAP. Immature tubers are defined as tubers harvested between ca. 60–80 DAP and weighing less than 50 g fresh weight.

Certified (G3, certified generation three from nuclear stock) seed-tubers of 'Defender,' 'Umatilla Russet,' and 'Russet Burbank' were hand cut into 50- to 64-g seedpieces and suberized at 10°C ± 0.5 °C (95% RH) for 5 days prior to planting. The seedpieces were planted 20-cm deep in a Shano silt loam soil [48] in four, 90-m long rows spaced 86 cm apart with seedpieces 25 cm apart within each row [25]. Plots were managed according to standard practices for production of long-season russet potatoes in the Columbia Basin and plants and tubers were harvested at approximately 10-day intervals from 54 to 170 DAP to establish the developmental growth profiles (Fig. 2) as described [49]. Four replicates of four consecutive plants per replicate were selected at random from within the two center rows for each harvest. Specific gravity (a direct indicator of tuber dry matter and starch content) was quantified for a five-tuber sample of each replicate [49], starting 76 DAP. Foliar and tuber fresh wt. were plotted versus DAP to establish the post tuberization developmental profiles. Average tuber fresh wt. at each developmental stage (stages 1–10; Supplemental Table 1) were estimated from the second degree polynomials describing changes in tuber weight as a function of days after planting (Fig. 2). Tuber physiological maturity was calculated as the average of DAP to reach maximum yield, maximum tuber specific gravity, minimum sucrose, and minimum reducing sugars in the basal ends of tubers. Cumulative growing degree days are shown in Fig. 2 and some additional weather data can be found at the Ag WeatherNet System (weather.wsu.edu). Starch content can be estimated from the specific gravity data presented in Fig. 2 by using the Von Scheele method [50]. Samples from Russet Burbank, Umatilla Russet and Defender were prepared as described in Section 4.3. All other samples used powder prepared from whole potatoes that had been quartered, rapidly frozen in liquid nitrogen then freeze-dried.

4.3. Carbohydrate analyses

Twenty tubers were sampled at each harvest in four replicates of five tubers each. Tubers were cut in half longitudinally along the apical to basal axis. The tubers at each harvest were chosen to represent the average tuber fresh weight (g/tuber) corresponding to each of the ten stages of development depicted in Supplemental Table 1. A thin slice (approximately 1.5 mm thick, periderm attached) was taken from the cut surface of one of the tuber halves with an electric slicer (Sunbeam Products Inc., Boca Raton, FL). The longitudinal slice was then halved along the apical to basal axis and one half was retained to represent an individual tuber. Collectively, the longitudinal tissue slices from the five tubers making up each replicate were cut into apical and basal halves and immediately frozen at −80 °C. The tissue samples were lyophilized, ground, sieved through a 60-mesh (0.246 mm) screen, and stored until the end of the growing season when all samples had been collected and processed similarly.

Sucrose, glucose and fructose concentrations were quantified as described in Knowles et al. [51]. Briefly, 500 mg of lyophilized tissue was extracted in 6 ml of triethanolamine HCl (TEA) buffer (30 mM, pH 7.0) followed by successive additions of 300 μL of 85 mM $K_4[Fe(CN)_6]\cdot 3\cdot H_2O$ (Carrez I), 300 μL of 250 mM $ZnSO_4\cdot 7H_2O$

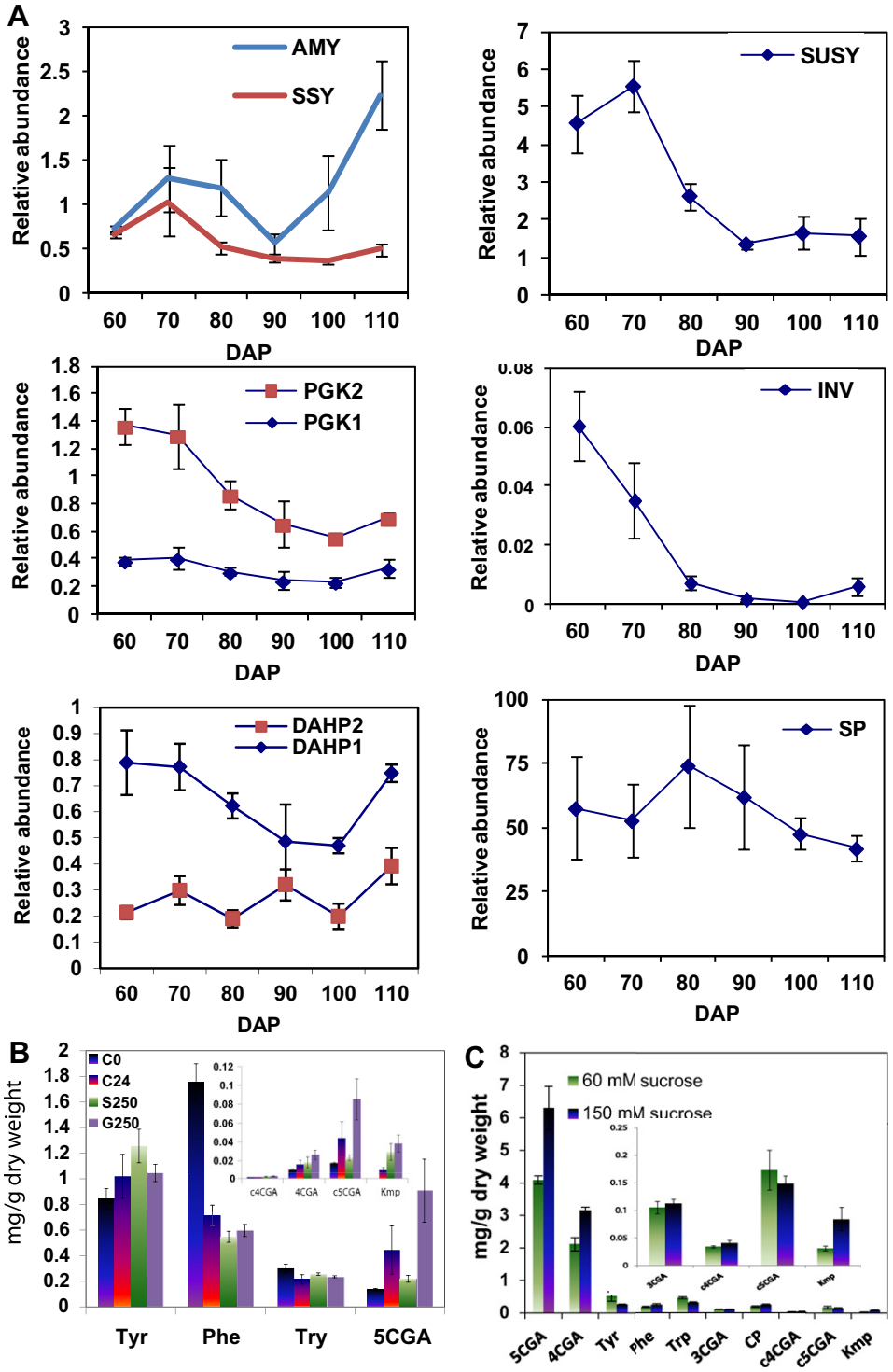

Fig. 9. Tuber carbohydrate metabolism. (A) Relative expression during tuber development of genes involved in starch metabolism, glycolysis or shikimic acid biosynthesis. 'Green Mountain' tubers were harvested at six developmental stages (DAP). Normalized qRT-PCR data represents the average of 3 biological replicates with two technical replicates each for each timepoint. DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase; PGK, phosphoglycerate kinase; AMY, alpha-amylase; SUSY, sucrose synthase; SSY, soluble starch synthase; INV, soluble acid invertase; SP, starch phosphorylase. (B) Phenylpropanoid content in tuber discs at time zero (C0); 24 h after slicing (C24); 24 h incubation with 250 mM sucrose (S250) or 250 mM glucose (G250). Inset shows less abundant compounds. (C) Compounds measured in potato plantlets grown on standard tissue culture medium or medium supplemented with 150 mM sucrose. Standard error is shown ($n = 3$).

(Carrez II), and 500 μL of 0.1 mM NaOH. The extract was centrifuged at 10,000*g* for 15 min and the supernatant was stored at −20 °C until analysis. Glucose and fructose were estimated according to Bergmeyer et al. [52] and Bernt et al. [53] using a modified microplate method [51]. The stoichiometric reduction of NADP as each hexose is converted to 6-phosphogluconate was monitored at A_{340}. Thirty μL of extract was added to 160 μL of 0.3 M TEA buffer (pH 7.4) containing 20.6 μM ATP, 1.6 μM NADP and 3 mM MgSO4 to

Fig. 10. Differences in phenylpropanoid expression in purple potatoes grown in the field (left) versus greenhouse (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

dilute glucose, fructose and sucrose to less than 1.0 mM each. For determination of free glucose, 10 nkat each of glucose-6-phosphate dehydrogenase and hexokinase were added simultaneously and A_{340} was measured after 15 min incubation. Phosphoglucose isomerase (17 nkat) was then added and the extracts were incubated for 30 min before measuring A_{340} for the quantitation of fructose. A separate sample of the extract was pre-treated for 30 min with 14 nkat invertase in 0.1 M acetate buffer (pH 4.6) for the determination of total glucose (free glucose plus that hydrolyzed from sucrose via invertase). The difference between moles total glucose and moles free glucose was used as a measure of sucrose [54]. All steps were carried out at room temp. Quantitation was based on standard curves of glucose, fructose and sucrose (0.05–2.4 mM).

4.4. LCMS analysis of phenylpropanoids

Freeze-dried tissue (200 mg for white potatoes or 100 mg for colored-flesh potatoes) was extracted with 0.9 ml of extraction buffer (50% MeOH, 2.5% metaphosphoric acid, 1 mM EDTA) and 500 mg of 1.0 mm glass beads and shaken in a BeadBeater (Biospec, Bartelsville, OK) for 15 min at maximum speed, then centrifuged for 5 min at 4 °C. The remaining pellet was reextracted with 0.6 ml of extraction buffer and the supernatants combined, centrifuged and concentrated in a Speed Vac (Thermo Savant, Waltham, MA) prior to LCMS analysis. Samples and solutions were kept chilled at all times and not exposed to bright light.

An Agilent 1100 HPLC system equipped with a quaternary pump, refrigerated autosampler, and column heater was used with DAD and MS detectors. A 100 × 4.6 mm, Onyx monolithic C-18 (Phenomenex) was used at a column temp of 35 °C and flow rate of 1 mL/min with a gradient elution of 0–1 min 100% A, 1–9 min 0–30% B, 9–10.5 min 30% B, 10.5–14 min 35–65% B, 14–16 min at 65–100% B, 16–16.5 min 100% B (Buffer A: 10 mM formic acid pH 3.5 with NH_4OH; Buffer B: 100% methanol with 5 mM ammonium formate). UV detection was at 210, 244, 280, 320 and 360 nm. MS analysis was with an Agilent 1100 LC/MSD VL or SL ion trap using an ESI source in both positive and negative ion mode. As necessary to optimize MS^2 data collection, the target mass, trap drive level and compound stability were modified. The source was operated using 350 °C drying gas (N_2) at 12 L/min, 55 psi nebulizer gas (N_2), and the source voltage with a scan range of m/z 100–1250 or 80–900. Data analysis was performed using Agilent ChemStation software. The external standard method of calibration was used. CGA isomers were quantitated as chlorogenic acid equivalents with gallic acid as an internal standard [55]. If a compound could not be quantitated with the DAD, whether due to co-elution or low abundance, relative amounts were determined with the ion trap. When standards were commercially available, identification was based on retention time and MS data, or tentative assignments were made based on UV and MS data when standards were not available. Retention times and MS data for compounds analyzed are shown in Supplemental Table 2. MS^2 ions are listed in order of abundance and relative amounts are shown only for ions used to discriminate among possible isomers [56].

4.5. RNA extraction and cDNA synthesis

RNA was extracted from freeze-dried tuber samples using the CTAB (cetyltrimethylammonium bromide) method [57]. Briefly, 1 ml of CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 0.1 M Tris–HCl pH 8.0, 2% w/v PVP (K-30), 0.2% v/v β-mercaptoethanol) was added to 50 mg dry powder and vortexed for 3 min. Seven hundred μl of phenol:chloroform:isoamyl alcohol (125:24:1) was then added, vortexed for 2 min, incubated for 5 min at 65 °C and centrifuged for 10 min at 13,000g. The supernatant was again extracted with 600 μl of chloroform:isoamyl alcohol (24:1). After adding 400 μl of 8 M LiCl, samples were incubated overnight at 4 °C, and RNA precipitated by centrifuging for 10 min at 4 °C. The pellet was dissolved in 100 μl of dH_2O, 1 μl DNAse enzyme added (New England BioLabs, Ipswich, MA), incubated for 30 min at 37 °C, and the DNAse inactivated by heating at 65 °C for 20 min. Two hundred μl of dH_2O, 30 μl of 3 M sodium acetate (pH 5.2) and 750 μl of 95% ethanol was then added and the solution incubated at −80 °C for 1 h and centrifuged at 4 °C for 20 min. RNA quantity was assessed by using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was assessed by running 375 ng RNA on a 1% agarose gel. cDNA was synthesized using 2 μg total RNA, anchored oligo (dT) 20VN primers and M-MuLV reverse transcriptase (New England BioLabs).

4.6. QRT-PCR expression analysis

Relative transcript abundance was analyzed by qRT-PCR in a 12 μl reaction volume using 4 ng RNA equivalent cDNA, 400 nM gene-specific primers and 6 μl SYBR Green Mix (Roche, Mannheim, Germany). A 5 min preincubation at 95 °C was followed by 40 cycles of 10 s denaturation at 95 °C, 20 s annealing at 60 °C, and 20 s extension at 72 °C, using a Lightcycler 480 (Roche). Three biological and two technical replicates were used for each determination. Relative expression of the genes was calculated by the ΔC_T method [58] by normalizing the C_T levels of target genes to the geometric mean of C_T levels of two housekeeping genes, actin and elongation factor (EF1-α). Specificity of amplification was assessed by dissociation curve analysis and the PCR product analyzed on a gel. Primer sequences (Supplemental Table 2) for EF1-α, PAL, C4H, C3H, HQT and CHI, were from Andre et al., 2009 or potato genome sequence data [59]. Data analysis was performed in Microsoft Excel and a heatmap generated with Heatmapper Plus, using Pearson correlation coefficients [60].

4.7. Sugar feeding experiments

For plantlet experiments, 1 month old tissue culture plants were subcultured in basal media (4.3 g l^{-1} MS salts, 0.1 g l^{-1} myoinositol, 0.25 mg l^{-1} folic acid, 0.05 mg l^{-1} d-biotin, 2 mg l^{-1} glycine, 0.5 mg l^{-1} nicotinic acid, 0.5 mg l^{-1} pyridoxine, 0.4 mg l^{-1} thiamine HCL, 60 mM sucrose (normal formulation) or 150 mM sucrose, pH 5.8, solidified with 3.7 gm l^{-1} gelite). The plants were cultured at 25 °C under fluorescent lights with 16 h and 8 h light and dark cycle, respectively. After 14 days, plantlets were frozen in liquid nitrogen, freeze-dried, ground and stored in −80 °C until used. For

tuber disc assays, small tubers were washed with distilled water and a tuber core removed with a 1.3-mm cork borer and cut into 2 mm thick disks. The discs were distributed evenly in flasks containing either buffer (2.5 mM MES, pH 6.5), sucrose (50 and 250 mM) or glucose (250 mM). The discs were incubated with gentle shaking in the dark. After 24 h, the disks were rinsed in dH_2O, frozen in liquid nitrogen, freeze-dried, ground and stored in −80 °C until used. Control samples (time zero) were collected and frozen immediately at the start of the experiment.

4.8. HQT transient assay

The HQT sequences of potato (genebank accession no. DQ200363), tomato (accession no. AJ582652) and tobacco (accession no. AJ582651) were aligned and the consensus sequence was used to design PCR primers for amplification of cDNA in Norkotah. Roughly 400 bp of HQT gene was cloned in pHellsgate8 vector and transformed into agro strain LBA4404. The recombinant bacteria were selected on LB medium supplemented with 25 mg mg^{-1} rifampicin and 50 mg mg^{-1} spectinomycin. The overnight culture was centrifuged, resuspended in infiltration buffer (10 mM $MgCl_2$ and 100 μM acetosyringone) and OD adjusted to 0.6. The culture was infiltrated into leaves using a syringe and the leaves were harvested after 7 days. An empty vector served as a negative control.

4.9. Enzyme and phenolic assays

Soluble proteins were extracted from 50 mg freeze dried powder with 750 μl of cold 50 mM Tris–HCl (pH 8.8) containing 1 mM EDTA, 1 mM PMSF, 5.7 mM β-mercaptoethanol, 1% insoluble PVPP, and 0.2% Triton X-100. Protein concentration was estimated using Bradford reagent with *bovine serum albumin* (BSA) as standard. PAL was measured as described [61] with modifications. A 1 ml reaction contained 100 μl of the protein extract, 10 mM phenylalanine and 50 mM final concentration of sodium borate buffer (pH 8.8). Samples were incubated for 1 h at 37 °C. The reaction was stopped by adding 200 μl of 12% trichloroacetic acid, centrifuged for 10 min, and A_{290} measured.

For the assay of PPO, HQT and shikimate dehydrogenase, 50 mg of freeze-dried tuber tissue was extracted twice with 500 μl of 50 mM potassium phosphate buffer pH 7.0 containing 2% PVPP (w/v), 1 mM EDTA, 1 mM DTT and 3 μl of protease inhibitor cocktail. The supernatant was adjusted to 30% ammonium sulfate with 1% PVPP (w/v). Extracts were mixed at 4 °C for 30 min then centrifuged at 13,000*g* for 15 min. The supernatant was adjusted to 80% ammonium sulfate, shaken at 4 °C for 30 min and centrifuged at 13,000*g* for 15 min. The pellet was resuspended in 130 μl of assay buffer (50 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA) and desalted using a Zebra spin column (Thermo Scientific, Rockford, IL, USA). The eluent was adjusted to 200 μl with buffer and 1 μl protease inhibitor.

The HQT reverse reaction was measured at A_{360} [62] for 2 min in a BioTek Synergy 2 microplate reader at 30 °C. A 100 μl reaction in 50 mM phosphate buffer pH 7.0 contained 60 nmoles CoA and 50 nmoles chlorogenic acid. Shikimate dehydrogenase was assayed in 100 μl of 50 mM Tris–HCl pH 8.0 containing 4 mM $MgCl_2$, 1 mM NADP and 2 mM shikimate and the increase in A_{340} was measured for 5 min at 30 °C [63]. PPO was assayed at A_{420} in 100 μl of 50 mM phosphate buffer, pH 7.0 with 25 mM catechol for 5 min at 30 °C.

Total phenolics were measured using Folin–Ciocalteu reagent (FC) in a modified protocol [64]. One hundred microliters of extract and 1.5 ml water were mixed with 100 μl FC reagent and incubated for 3 min at room temp. After adding 300 μl of 20% (w/v) Na_2CO_3, the sample was incubated 30 min at 40 °C and the A_{755} recorded. Lignin was measured according to Whitmore [65].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2013.01.007.

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